Inducible antisense RNA targeting amino acid transporter ATB\textsuperscript{0}/ASCT2 elicits apoptosis in human hepatoma cells

Bryan C. Fuchs, J. Christian Perez, Julie E. Suetterlin, Sofia B. Chaudhry and Barrie P. Bode

Department of Biology, Saint Louis University, St. Louis, Missouri 63103-2010

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The amino acid glutamine is avidly utilized by multiple cell types in the body as a metabolic intermediate, contributing carbon and nitrogen for the synthesis of other amino acids, fatty acids, nucleic acids, and proteins, and it also serves as an oxidizable substrate for ATP production in dividing cells (22). Its pivotal role in cellular metabolism is underscored by its relatively high turnover rate in all cell types (12) and by the fact that it is the most abundant amino acid in plasma at levels around 0.6 mM. Glutamine also serves as the primary nonoxidative shuttle for ammonia from extrahepatic tissues to the liver, where it is taken up by the amino acid transporter System N (20) and utilized in the urea cycle, transamination reactions, and gluconeogenesis (27). On the basis of the unique metabolic heterogeneity along the liver acinus, the liver can rapidly alter flux through glutamine-producing or -consuming pathways and thus serves as a systemic integrating center for glutamine homeostasis (15).

In hepatocellular carcinoma (HCC), net glutamine consumption is observed with utilization of this nitrogen-rich amino acid for growth-related pathways (10). Consequently, the systemic glutamine homeostatic function of the liver is subverted, resulting in significantly decreased plasma glutamine levels in patients with liver cancer (16), an event that can adversely impact glutamine-dependent cells, such as those in the immune system (1). Previous work from our laboratory (6–9) showed that human hepatoma cells take up glutamine at rates 10- to 30-fold faster than normal human hepatocytes via a transporter historically termed System ASC. This high-affinity Na\textsuperscript{+}-dependent glutamine transport activity is not expressed in normal hepatocytes (7). The system ASC transporter was later cloned and termed amino acid transporter B\textsuperscript{0} (ATB\textsuperscript{0}) (19) and seems to be the human ortholog of rodent ASC transporter 2 (ASCT2) (5). This transporter also has been identified as a cell surface receptor for mammalian retroviruses and is referred to as human ASCT2 (35). ATB\textsuperscript{0}/ASCT2 expression was recently reported to be absent in normal human hepatocytes but present in six human hepatoma cell lines and primary liver tumors and shown to mediate the glutamine-dependent growth of rapidly growing human hepatoma cells (6). These results were confirmed by a recent query at the Cancer Genome Anatomy Project website (http://cgap.nci.nih.gov) that showed no ATB\textsuperscript{0}/ASCT2 (accession #U53347) expression in normal human liver [0/34,164 expressed sequence tags (ESTs)] but appreciable expression in liver cancer (17/40,737 ESTs) using the Virtual Northern program. Thus expression of this transporter is activated in human liver cancer.

According to the American Liver Foundation, HCC is the most common primary malignant tumor of the liver. HCC is also the leading cause of cancer death in Africa and the developing world (28), and its prevalence in Europe and the United States is on the rise due to increased incidence of viral hepatitis. Currently, there is no effective treatment for HCC other than resection or transplant, and both modalities are often unsuccessful. Given the glutamine reliance of aggressively growing hepatoma cells, we hypothesized that inhibition of ATB\textsuperscript{0}/ASCT2 expression would arrest the growth of human hepatoma cells. Because this transporter is not expressed in normal hepatocytes, we hypothesized that its expression is necessary for SK-Hep cell growth and viability and suggest that it be further explored as a selective target for human hepatocellular carcinoma.


Address for reprint requests and other correspondence: B. P. Bode, St. Louis Univ., Dept. of Biology, 3507 Laclede Ave., St. Louis, MO 63103-2010 (E-mail: bodebp@slu.edu).
of several human hepatoma cell lines examined (6) and rapidly forms highly vascularized tumors when injected subcutaneously into athymic nude mice (Bode, unpublished data and Ref. 18). Second, SK-Hep cells possess the highest glutamine uptake velocity of all human hepatoma cell lines examined (6), rates that are further enhanced by three-dimensional growth in vitro (30). Finally, SK-Hep cells exhibit accelerated glutaminase rates (9) and decreased glutamine synthetase expression (6) similar to clinical HCC (25). Previous studies (38) have shown that SK-Hep cells initially respond to glutamine deprivation by increasing the affinity of ATB0 for glutamine but do not survive in glutamine-free medium beyond 72 h. Furthermore, competitive inhibition of ATB0 glutamine transport with other ATB0/ASCT2 amino acid substrates arrests the growth of SK-Hep cells (8). On the basis of these observations, the purpose of this study was to test the hypothesis that selective inhibition of ATB0/ASCT2 would lead to growth arrest of this aggressively growing human liver cancer cell. To this end, we constructed an inducible antisense RNA-based expression system that selectively targets the ATB0/ASCT2 mRNA and mediates its degradation. Results indicate that the differential expression of this transporter in transformed/cancerous liver cells may be essential for their growth and survival.

MATERIALS AND METHODS

Cell culture. The human hepatoma cell line SK-Hep1 (American Type Culture Collection, Rockville, MD) was maintained at 37°C in a humidified atmosphere of 5% CO2-95% air in DMEM (4.5 mg/ml d-glucose) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 µg/ml streptomycin (all from Invitrogen Life Technologies, Carlsbad, CA). SK-Hep cells stably transfected with both pSwitch and pGene/V5-HisATB0 (described in Generation of stably transfected hepatoma cells) were maintained in growth media supplemented with 300 µg/ml hygromycin B and 200 µg/ml Zeocin (both from Invitrogen Life Technologies). For glutamine deprivation studies, cells were grown in DMEM supplemented with 2 mM L-glutamine, containing 10% dialyzed FBS, 100 U/ml penicillin G, and 100 µg/ml streptomycin and supplemented with hygromycin B and Zeocin in the case of stably transfected clones.

Inducible antisense expression system. The GeneSwitch Inducible Mammalian Expression System (Invitrogen Life Technologies) was utilized for this study. The system involves a set of two vectors stably maintained by antibiotic selective pressure. The gene of interest is expressed in the pGene/V5-HisA vector which contains a hybrid promoter sequence consisting of six binding sites for the yeast GAL4 protein and a 10-bp TATA box sequence from the Adenovirus E1b gene. Until induced, the promoter is transcriptionally silent allowing for extremely low levels of basal expression. The second vector, pSwitch, encodes for the GeneSwitch chimeric transcription factor that activates transcription from the pGene/V5-HisA vector containing the gene of interest. The GeneSwitch protein has three functional domains including a GAL4 DNA binding domain, a human progesterone receptor ligand-binding domain (hPR-LBD) that binds the inducing agent mifepristone (MFP), and an NF-κB p65 activation domain to activate transcription from the GAL4 UAS/E1b minimal promoter of pSwitch. In the absence of MFP, the conformation of the hPR-LBD region prevents the GeneSwitch protein from activating transcription. MFP, also known as RU-486, is a synthetic 19-nortestosterone that normally acts as both a progesterone and glucocorticoid antagonist but at the low concentration used in this system acts as an agonist, binding the hPR-LBD region of the GeneSwitch protein, inducing a transcriptionally active conformation.

Construction of the inducible antisense RNA vector. The human ATB0 cDNA, kindly provided by Dr. Vadivel Ganapathy (Medical College of Georgia) was isolated from the pSPORT1 vector by a double restriction enzyme digestion using KpnI and NotI (both from Promega, Madison WI). The full-length ATB0 cDNA and the pGene/V5-HisA vector were each subsequently digested at 37°C overnight with Apal (Promega). Apal digestion of the ATB0 cDNA yields fragments of 107, 170, 218, 569, and 1,333 bp. The 1,333-bp fragment of ATB0 represents base pairs 872–2205, spanning 82% of the coding sequence (base pairs 620–2245). Apal-linearized pGene/V5-HisA vector was treated with shrimp alkaline phosphatase (Promega) followed by ligation with the gel-purified 1.3-kb ATB0 fragment at 4°C overnight, using T4 DNA ligase (Promega). The resultant 5.9-kb pGene/V5-HisATB0 plasmid was subsequently transformed into Escherichia coli-competent cells, amplified, and purified (Concert MaxiPrep, Invitrogen Life Technologies). Because only Apal was used for the digestion, BamHI restriction digests of the 5.9-kb constructs were utilized to assess the orientation of any particular clone. Inserts were additionally confirmed by CEQ dye terminator cycle DNA sequencing (Beckman-Coulter, Fullerton, CA) with a Beckman CEQ 2000 capillary sequencer using primers specific for the pGene/V5-HisA multiple cloning site. These constructs, designated as pGene/V5-HisATB0 Antisense and pGene/V5-HisATB0 Sense, were used to generate stably transfected SK-Hep cells.

Generation of stably transfected hepatoma cells. SK-Hep cells were transduced into six-well plates (Corning, Cambridge, MA) at a concentration of 1 x 105 cells/ml, were allowed to adhere to the plates overnight, and were ~30% confluent for transfections the next day with Lipofectamine in OptiMEM serum-free media (both from Invitrogen Life Technologies), at a ratio of 8 µL Lipofectamine/1 µg FspI-linearized pSwitch DNA. Transfections were performed by standard manufacturer-recommended procedures, and 48 h later, the cells were removed by trypsinization and transferred into 150-mm tissue culture plates (Corning, Acton, MA). Cells were maintained in selective growth media supplemented with 300 µg/ml hygromycin B, a concentration empirically determined by titration analysis. Individual colonies were isolated 7–10 days later using sterile cloning cylinders (Sigma, St. Louis, MO) and maintained in selective growth media. A pGene/V5-HislacZ reporter gene vector was used to identify individual colonies that exhibited the highest inducibility with the lowest levels of basal expression, via transient transfection and X-Gal (blue) staining. Appropriately stably transfected pSwitch SK-Hep clones were selected, one of which was utilized to generate the double stably transfected antisense and sense clones used in this study.

pSwitch SK-Hep cells were transfected with either pGene/V5-HisATB0 Antisense or pGene/V5-HisATB0 Sense constructs. Colonies resistant to the pGene/V5-His selectable marker Zeocin at an empirically determined concentration of 200 µg/ml were isolated. Several sense and antisense pGene/V5-HisATB0 clones were screened for their response to induction with MFP. These stably transfected SK-Hep clones were maintained in selective media containing 300 µg/ml hygromycin B and 200 µg/ml Zeocin.

RNA isolation and Northern blot procedure. Total cellular RNA was isolated by the one-step acid phenol-guanidinium method and analyzed by Northern blotting analysis as previously described (6). Full-length 2.9-kb sense and antisense ATB0 3P-labeled RNA riboprobes were generated by in vitro transcription from the ATB0 cDNA in the pSPORT-1 vector using SP6 or T7 RNA polymerase (MAXIscript; Ambion, Austin, TX) and [α-32P]UTP (Amersham, Arlington Heights, IL). The pSPORT1 vector was linearized with HindIII (Promega) and transcribed with T7 RNA polymerase to generate the sense probe that detects induced antisense ATB0 RNA. Conversely, pSPORT1 was linearized with RsrI (New England Biolabs) and transcribed with the SP6 polymerase to make the antisense probe for detection of endogenous ATB0 mRNA. A 2.1-kb human ASCT2 cDNA (kindly provided by Dr. Mike Kilberg, University of Florida) was excised from pcDNA3 with HindIII and XhoI and used to generate a random hexamer-primed radiolabeled probe (Megaprime; Amersham) for Northern blot anal-
ysis. A pTRI-actin-mouse DNA template (250-bp KpnI-Xhol fragment) was transcribed in vitro with the SP6 polymerase to generate a 334-bp antisense β-actin riboprobe as a positive control (Ambion). Membrane hybridization and washing under high-stringency conditions was performed as previously described (6). Band intensities on X-ray film were quantified by using the Kodak EDAS 290 system with one-dimensional image analysis software (Eastman Kodak, New Haven, CT). Each Northern blot was repeated at least once to ensure reproducibility and to demonstrate qualitatively similar results.

**Results**

**Screening of stably transfected hepatoma cells.** SK-Hep cells stably transfected with the inducible transcription factor [Ac-DEVD-pNA (CaspACE, Promega)]

**Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining.** Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed with the DeadEnd colorimetric TUNEL system (Promega). Brieﬂy, cells were plated on FALCON culture slides (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 5 × 10^4 cells/ml and allowed to grow for 2 days, then treated with MFP or vehicle for 24 h. After treatment, slides were washed in PBS, fixed in 4% (wt/vol) paraformaldehyde, and stained according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). In these assays, caspases-2 (VDVAD), -8 (IETD), and -9 (LEHD) recognition sequence peptides conjugated to the chromophore para-nitroaniline (pNA) are cleaved by the activated enzymes, and the liberated pNA is quantified by spectrophotometric absorbance at 405 nm. The pNA released was normalized to total protein content in the lysates by the bicinchoninic acid (BCA) method (Pierce Chemical, Rockford, IL), and results are expressed as picomoles pNA released per microgram protein per hour. Likewise, effecter caspase-3 activity was analyzed with a chromogenic substrate [Ac-DEVD-pNA (CaspACE, Promega)].

**Western blot analysis.** Relative levels of intact and cleaved poly-(ADP-ribose) polymerase (PARP), total and phosphospeciﬁc, double-stranded RNA-dependent protein kinase R (PKR), were determined by Western blot analysis using rabbit polyclonal antibodies directed against PARP, PKR, and phosphothreonine (Thr^34,^46,^56) PKR (Cell Signaling Technology, Beverly, MA). Cellular lysates were prepared, separated by electrophoresis on 4–20% polyacrylamide gradient gels, transferred electrophoretically to polyvinylidene difluoride membranes, and incubated with primary antibodies in blocking buffer [5% nonfat dry milk, 0.1% Tween 20 in Tris-buffered saline (TBS)] overnight at 4°C. Blots were washed three times in wash buffer (0.1% Tween 20 in TBS). After a second incubation with a HRP-linked anti-rabbit IgG antibody for 1 h, immunoreactive bands were visualized on X-ray ﬁlm with a chemiluminescent HRP substrate (Phototope, Cell Signaling Technology). The molecular size of the detected bands was calculated from molecular weight standards resolved on the same gels as the experimental samples. Band intensities on X-ray ﬁlm were quantiﬁed using the Kodak EDAS 290 system with one-dimensional image analysis software (Eastman Kodak). Each Western blot was repeated at least once to ensure reproducible qualitatively similar results.

**Vital dye staining.** Visualization of apoptotic nuclei was further examined with acridine orange and ethidium bromide staining as described in detail (11).
Effect of antisense expression on ATB\textsuperscript{0}/ASCT2 mRNA levels. On induction with 10 nM MFP, a 1.3-kb antisense ATB\textsuperscript{0}/ASCT2 RNA complementary to the reading frame (620–2245) was detectable after 14 h in the Antisense 1-1 cells (Fig. 1), but by 24 h, antisense RNA levels waned. No MFP-induced antisense transcript was observed in Sense 2-1 cells or the parent SK-Hep cells (Fig. 1). Expression of the antisense RNA led to a decrease in cellular ATB\textsuperscript{0}/ASCT2 mRNA levels of ∼85 and 73\% after 14 and 24 h, respectively, after normalization to β-actin levels in Antisense 1-1 (Fig. 2). This decrease was only observed in the Antisense 1-1 cells treated with MFP and not the vehicle control (0.1\% ethanol). In contrast, Sense 2-1 cells treated with MFP or ethanol showed no change in endogenous ATB\textsuperscript{0}/ASCT2 mRNA levels (Fig. 2). These results indicate that cells expressing the 1.3 kb antisense RNA exhibit markedly lower ATB\textsuperscript{0}/ASCT2 mRNA levels compared with controls. To further investigate the specificity of the response, ASCT1 mRNA levels were also investigated. ASCT1 is 63\% homologous to ASCT2 in the region targeted by the 1.3-kb antisense RNA. Although this System ASC isoform does not take up glutamine, it shares considerable substrate overlap with ASCT2 (2, 33). The results in Fig. 2 show that ASCT1 mRNA levels are markedly enhanced by ASCT2 knockdown after 14 and 24 h.

Impact of antisense ATB\textsuperscript{0}/ASCT2 RNA on glutamine transport rates. To determine the functional effect of targeting ATB\textsuperscript{0}/ASCT2 mRNA, glutamine transport rates were measured. Sodium-dependent glutamine uptake rates revealed significant (\(P < 0.010\)) reductions of 49 and 65\% in the Antisense 1-1 cells after induction with MFP for 14 and 24 h, respectively (Fig. 3). There were no significant differences in glutamine transport rates after induction for the control Sense 2-1 cells. To determine whether ASCT2 was still responsible for the residual glutamine uptake observed after its suppression, amino acid inhibition analysis was performed (Fig. 3C). After transporter knockdown, glutamine uptake remained largely sodium dependent and was mediated almost entirely by ASCT2 on the basis of significant (\(P < 0.010\)) inhibition by its substrate, threonine. Threonine inhibited glutamine uptake by 92 and 93\% in the MFP controls after 14 and 24 h, respectively, and 93 and 88\% after antisense ATB\textsuperscript{0}/ASCT2 expression (+MFP) for 14 and 24 h, respectively (Fig. 3C). No significant (\(P < 0.010\)) decreases in glutamine uptake were observed in the presence of MeAIB (System A), although MeAIB may have had a slight \textit{cis}-stimulatory effect on glutamine uptake in all cases as illustrated in Fig. 3C. Arginine [Systems A (via ATA3), B\textsuperscript{0\%}, y\textsuperscript{+}, or y\textsuperscript{+L}] inhibited glutamine

![Fig. 2. Northern blot analysis of endogenous ATB\textsuperscript{0}/ASCT2 mRNA expression.](image-url)
uptake marginally (~20%), but this effect was only significant in cells treated with MFP for 14 h (Fig. 3C). System N is not expressed by SK-Hep cells (6), and the degree of nonsaturable uptake was not enhanced on the basis of the marked inhibitory effect of unlabeled glutamine (97% for all treatments). Taken together, the results indicate no compensatory glutamine uptake response to ASCT2 suppression and that the residual activity reported in Fig. 3B is entirely attributable to ASCT2.

Effect of ATB0/ASCT2 antisense RNA on cellular growth and viability. Corresponding with the decreases in transporter mRNA levels and glutamine transport rates, the Antisense 1-1 cells also exhibited growth arrest with subsequent cellular loss. Significant (P < 0.010) reductions in cell numbers of 17, 77, and 98% were observed at 14, 24, and 48 h after induction, respectively, relative to controls (Fig. 4A). In contrast, growth of the SK-Hep parent cell line and the Sense 2-1 controls was unaffected by treatment with MFP (data not shown). The reduction in cell number after induced ATB0/ASCT2 antisense RNA expression was attributed to cellular death and loss as determined by the lack of trypan blue exclusion (results not shown). Dead cells showed high levels of cell blebbing (Fig. 4B), a hallmark of apoptosis.

Role of glutamine deprivation in antisense RNA-mediated cell death. Studies presented here evolved from the central role of ATB0/ASCT2 in driving glutamine-dependent growth of this liver cancer cell line (6, 8). To determine what role glutamine deprivation plays in the cellular death of the Antisense 1-1 cells after induction with MFP, Antisense 1-1 cells were quantified after maintenance in glutamine-free media. Significant (P < 0.010) reductions in cell numbers of 27, 52, 80, and 94% relative to controls were observed at 14, 24, 48, and 72 h, respectively, after culture in the absence of glutamine (Fig. 4A). Glutamine deprivation thus required ~24 h more than ATB0/ASCT2 suppression to effect nearly complete cellular loss. One other notable difference between the two treatments was that glutamine-deprived cells exhibited marked stress fiber formation (between 24 and 48 h) before cell death, a feature not observed with MFP-treated Antisense 1-1 cells (Fig. 4B). Similar to antisense RNA-induced cellular death, however, glutamine-deprived cells showed a high degree of cellular blebbing coupled with cellular loss (Fig. 4B). On the basis of these results, it is concluded that glutamine deprivation may contribute but alone is not sufficient to account for the effects of transporter knockdown.

Characterization of hepatoma cell death. To investigate the possible stimulation of apoptotic cascades by transporter knockdown or glutamine starvation, we measured the activity of effector caspase-3 and the cleavage of one of its well-characterized targets, PARP. The specific activity of caspase-3 was 3.3-fold higher in Antisense 1-1 cells induced with MFP after 24 h relative to the 0.1% ethanol-treated controls (Fig. 5A). No significant increases in caspase-3 activity were observed in the Sense 2-1 cells treated with MFP; in fact, a 48% reduction in caspase-3 activity was noted in MFP-treated cells relative to 0.1% ethanol-treated controls (Fig. 5A). For comparison, glutamine deprivation increased caspase-3-specific activity in Antisense 1-1 cells by 1.6-, 2.0-, and 4.1-fold after 24, 36, and 48 h of glutamine deprivation, respectively (Fig. 5B). These results indicate that both antisense ATB0/ASCT2 RNA and glutamine deprivation lead to activation of caspase-3.

To further assess the role of apoptosis in cell death elicited by transporter knockdown or glutamine deprivation, PARP cleavage in experimental samples was assessed by Western blot analysis. This DNA repair enzyme is one of the main targets of caspase-3 and is cleaved from its native molecular weight of 116 kDa to fragments of 89 and 24 kDa (36). The ratio of the p89 band intensity to the p116 band intensity was assessed by image analysis. MFP induction resulted in a twofold increase in this ratio after 24 h in Antisense 1-1 cells; conversely, no significant increase was noted in Sense 2-1 cells (Fig. 6). Glutamine deprivation caused a more dramatic PARP cleavage response but, similar to its effect on cellular death, was delayed relative to transporter knockdown. Whereas a marginal 14% increase in the p89-to-p116 PARP ratio was noted after 24 h of glutamine deprivation, a marked 5.3-fold
increase was noted after 48 h (Fig. 6). The relatively protracted temporal relationship of glutamine deprivation to PARP cleavage is consistent with the progressive activation of caspase-3 over 48 h (Fig. 5B).

Similarly, because nuclear fragmentation and caspase-dependent DNase activity is increased during apoptosis, we examined this endpoint via TUNEL analysis to assay for DNA cleavage in the Antisense 1-1 cells induced with MFP, because they showed a 3.3-fold increase in caspase-3 activation but only a twofold PARP cleavage increase after 24 h. Antisense 1-1 cells cultured in the presence of MFP for 48 h showed marked levels of TUNEL-positive cells compared with the vehicle controls (Figs. 7, A–D). These TUNEL-positive cells exhibited several distinct circular locations of intense brown staining, suggesting that the nuclei in these cells had been fragmented. In contrast, only a few random TUNEL-positive cells were seen in the SK-Hep parent cells and Sense 2-1 controls regardless of treatment with either MFP or ethanol (data not shown). Further studies employing vital dye staining with acridine orange and ethidium bromide revealed a progressive nuclear fragmentation pattern in MFP-treated Antisense 1-1 cells as well. Acridine orange is a vital dye that permeates and stains both living and dead cells (green), whereas ethidium bromide permeates and stains nuclei (bright orange) only in cells in which the membrane integrity is compromised (i.e., dying cells). No profound nuclear staining was evident 14 h after MFP induction in Antisense 1-1 cells, but after 24 h, bright green dots (acridine orange) were readily visible due to
obtained, and the specific activity of caspase-3 was calculated as described in MATERIALS AND METHODS. Results represent the average ± SD of 3 separate determinations in 1 experiment. Similar results were obtained in 2 additional studies. **P < 0.010 vs. −MFP or +GLN.

Caspase activation by transporter knockdown or glutamine starvation. To further investigate the mechanism linking ATB0/ASCT2 silencing to apoptotic cell death, initiator caspase activities were measured in cellular extracts for the intrinsic (caspase-9) and extrinsic (caspase-8) pathways after MFP treatment. The results shown in Fig. 8 indicate that the intrinsic pathway largely mediates ATB0/ASCT2 antisense RNA-mediated cell death. Marginal increases in caspase-8 activities of 15 and 27% (P < 0.010) were observed after 14 h and 24 h of MFP treatment, respectively, in Antisense 1-1 cells. No significant changes in caspase-8 activities were observed in cellular extracts from Sense 2-1 controls treated with MFP. In contrast, 2.5- and 1.6-fold increases in caspase-9 activities were observed in MFP-treated Antisense 1-1 cells after 14 and 24 h, respectively. Again, no significant changes were measured in caspase-9 activities in cellular extracts from Sense 2-1 control cells treated with MFP, except for a modest 21% increase after 24 h (P < 0.010). Caspase-2 bears features of both initiator and effector caspases and has been implicated in glutamine starvation-induced apoptosis in enterocytes (29). Caspase-2 activities were largely unaffected by MFP treatment in Sense 2-1 controls, except for a marginal 29% increase after 24 h. In contrast, caspase-2 activities were markedly stimulated in Antisense 1-1 cells in which increases of 3.2- and 2.7-fold were observed after 14 and 24 h of induction, respectively (Fig. 8).

For comparison, glutamine deprivation failed to stimulate either caspase-8 or -9 activities and induced caspase-2 activity after 48 h in Antisense 1-1 cells (Fig. 9). In fact, glutamine deprivation elicited decreases in caspase-8 activities of 20, 32, and 41% after 14, 24, 36, and 48 h, respectively (P < 0.010). Likewise, decreases in caspase-9 activities of 40, 47, and 29% (P < 0.010) were noted in cellular extracts after 24, 36, and 48 h of glutamine deprivation, respectively. Caspase-2 activity was significantly stimulated in glutamine-deprived cells only after 48 h, in which an increase of 72% (P < 0.010) was measured. Collectively, these results indicate that the initiating events leading to apoptosis differ between transporter knockdown and glutamine starvation.

Possible role of a PKR response in antisense RNA-mediated hepatoma apoptosis. Given the marked effect of induced ATB0/ASCT2 antisense RNA expression on hepatoma viability and apoptosis, the possibility that the transporter knockdown response was a nonspecific double-stranded RNA-dependent PKR-mediated event was considered. PKR mediates antiviral and stress responses in mammalian cells by phosphorylating eukaryotic initiation factor-2α and inhibiting viral and stress responses in mammalian cells by phosphorylating eukaryotic initiation factor-2α.

chormatin condensation and nuclear fragmentation; some ethidium bromide costaining was evident at this time point as well (Fig. 7E). After 48 h, the few remaining cells were readily observed to stain coincidentally with both dyes, indicative of late apoptotic cells.

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Fig. 5. Effect of antisense RNA expression and glutamine deprivation on caspase-3 activation. Cultures of Sense 2-1 (S 2-1) or Antisense 1-1 cells were treated at time 0 with inducer (10 nM MFP) or vehicle (0.1% ethanol) (A) or maintained in the presence or absence of glutamine (DMEM + 10% dialyzed FBS ± 2 mM l-GLN) (B). At the indicated times thereafter, cell lysates were obtained, and the specific activity of caspase-3 was calculated as described in MATERIALS AND METHODS. Results represent the average ± SD of 3 separate determinations in 1 experiment. Similar results were obtained in 2 additional studies. **P < 0.010 vs. −MFP or +GLN.

Fig. 6. Western blot analysis of poly(ADP-ribose) polymerase (PARP) cleavage after antisense induction or glutamine deprivation. Antisense 1-1 and Sense 2-1 cells were induced with MFP or grown in the absence of GLN for 24 or 48 h before preparation of cellular lysates. The level of PARP cleavage was determined by Western blot analysis with a PARP antibody that detects both the intact 116 kDa protein and the cleaved 89-kDa fragment. Band intensities were quantified using the Kodak EDAS 290 system with one-dimensional image analysis software, and the ratio of cleaved-to-intact PARP (p89/p116) was calculated. For each experimental condition, the ratio of the age after antisense induction or glutamine deprivation. Antisense 1-1 and Sense 2-1 control cells treated with MFP, except for a modest 21% increase after 24 h (P < 0.010). Caspase-2 bears features of both initiator and effector caspases and has been implicated in glutamine starvation-induced apoptosis in enterocytes (29). Caspase-2 activities were largely unaffected by MFP treatment in Sense 2-1 controls, except for a marginal 29% increase after 24 h. In contrast, caspase-2 activities were markedly stimulated in Antisense 1-1 cells in which increases of 3.2- and 2.7-fold were observed after 14 and 24 h of induction, respectively (Fig. 8).

For comparison, glutamine deprivation failed to stimulate either caspase-8 or -9 activities and induced caspase-2 activity after 48 h in Antisense 1-1 cells (Fig. 9). In fact, glutamine deprivation elicited decreases in caspase-8 activities of 20, 32, and 41% after 14, 24, 36, and 48 h, respectively (P < 0.010). Likewise, decreases in caspase-9 activities of 40, 47, and 29% (P < 0.010) were noted in cellular extracts after 24, 36, and 48 h of glutamine deprivation, respectively. Caspase-2 activity was significantly stimulated in glutamine-deprived cells only after 48 h, in which an increase of 72% (P < 0.010) was measured. Collectively, these results indicate that the initiating events leading to apoptosis differ between transporter knockdown and glutamine starvation.

Possible role of a PKR response in antisense RNA-mediated hepatoma apoptosis. Given the marked effect of induced ATB0/ASCT2 antisense RNA expression on hepatoma viability and apoptosis, the possibility that the transporter knockdown response was a nonspecific double-stranded RNA-dependent PKR-mediated event was considered. PKR mediates antiviral and stress responses in mammalian cells by phosphorylating eukaryotic initiation factor-2α and inhibiting viral and stress responses in mammalian cells by phosphorylating eukaryotic initiation factor-2α.
Fig. 7. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and Vital dye staining after induced antisense RNA expression. Antisense 1-1 cells were grown on FALCON culture slides and treated with MFP or vehicle for 24 h. After treatment, slides were washed in PBS, fixed in 4% paraformaldehyde, and subjected to TUNEL staining as described in MATERIALS AND METHODS. Slides were observed under light microscopy, and pictures were taken with a Kodak DC290 digital camera at magnifications of A: MFP, ×100; B: +MFP, ×100; C: +MFP, ×400; and D: +MFP, ×600. E: Vital dye staining of AS 1-1 after 24 or 48 h of treatment with MFP or vehicle. Cells were incubated with acridine orange and ethidium bromide, each at 4 μg/ml in PBS. Acridine orange staining was visualized through a FITC filter and ethidium bromide through a TRITC filter on a Nikon E400 epifluorescence microscope. Images of the same field for each time and condition are shown and were captured by the Kodak MDAS system. P/C, phase/contrast. Magnification for 24 h ±MFP is ×200 and for 48 h +MFP is ×400, illustrating a late apoptotic cell with fragmented nuclei.
protein synthesis (39). To this end, cells were treated with poly(IC), a mimic of double-stranded RNA and stimulator of PKR phosphorylation and activity in mammalian cells (39). When induced with MFP or treated with poly(IC), Antisense 1-1 cells failed to exhibit enhanced PKR phosphorylation above the rather significant baseline values present in controls (Fig. 10A), indicating that this enzyme is not further activated by antisense RNA-mediated transporter knockdown. Furthermore, results shown in Fig. 10B demonstrate that poly(IC) failed to significantly impact growth of the SK-Hep cells, except for a modest 17% decrease in MTT absorbance after 48 h. Taken together, these results suggest that the apoptosis elicited by ATB/ASCT2 suppression is not attributable to a nonspecific antiviral response but rather implicate an important role for this amino acid transporter in hepatoma growth and viability.

**DISCUSSION**

The studies presented here indicate that expression of the broad-scope Na⁺/and G475ASCT2 ANTISENSE RNA ELICITS HEPATOMA APOPTOSIS AJP-Gastrointest Liver Physiol • VOL 286 • MARCH 2004 • www.ajpgi.org
ASCT2 is essential for the growth and viability of SK-Hep human hepatoma cells. ATB\textsuperscript{0}/ASCT2 might serve as a selectively targeted therapy for HCC and that inhibition of its expression would lead to hepatoma growth arrest. The results presented here provide a proof of concept for that hypothesis, demonstrating that an aggressively growing human HCC cell undergoes apoptosis within 48 h after antisense RNA-mediated ATB\textsuperscript{0}/ASCT2 suppression. To the best of our knowledge, this is the first report of the therapeutic exploitation of a differentially expressed nutrient transporter in transformed vs. normal liver cells.

Antisense oligonucleotides have been successfully used by others to target glutamate transporters in the brain (32, 34) that belong to the same excitatory amino acid transporter family as ATB\textsuperscript{0}/ASCT2 (17). However, a stably maintained antisense RNA expression system was employed in this study to avoid issues of delivery/transfection efficiency associated with single-stranded oligonucleotides or small interfering double-stranded RNA molecules (siRNAs) used in RNA interference (RNAi) (14). Moreover, an inducible system was also employed, because we hypothesized that constitutive expression of transporter antisense RNA would be lethal to the SK-Hep cells. The results from this study confirm that suspicion. Targeting specific genes with long double-stranded RNA in mammalian cells often leads to a nonspecific antiviral response involving PKR and RNase L (4). However, lack of a PKR response in these cells (Fig. 10), coupled with no detectable degradation of total cellular RNA or β-actin and increased ASCT1 mRNA (Figs. 1 and 2), collectively argue against a nonspecific cellular response to ATB\textsuperscript{0}/ASCT2 suppression. There is also recent evidence that long double-stranded RNA within mammalian cells can be processed by mammalian dicer (40) into 21- to 23-nucleotide siRNA molecules and specifically suppress gene expression via RNAi (4). A sustained suppression of transporter mRNA (Fig. 2A), coupled with transient expression levels of antisense transcript over the first 24 h of induction (Fig. 1), further raises the possibility that ATB\textsuperscript{0}/ASCT2 knockdown may be mediated by RNAi in SK-Hep. However, more detailed studies will be required to test this hypothesis.

Initially, several clones stably transfected with ATB\textsuperscript{0}/ASCT2 antisense constructs were isolated, and most showed the same temporal kinetics of cell death after MFP induction as clone Antisense 1-1, which was selected for the more detailed studies presented in this report. Expression of the 1.3-kb antisense RNA caused a significant decrease in endogenous ATB\textsuperscript{0}/ASCT2 mRNA levels and sodium-dependent glutamine uptake rates at 14 and 24 h after induction (Figs. 2 and 3). It is difficult to assess the effects of antisense RNA on transporter protein turnover, because antibodies against the native transporter that are useful in immunoprecipitation or Western blot analyses have proven difficult to produce. Instead, glutamine uptake was measured, given that ATB\textsuperscript{0}/ASCT2 mediates over 90% of glutamine uptake into SK-Hep cells at the initial rate concentrations (50 μM) used in the transport assays (6, 7). With the use of this functional assay as an index of plasma membrane ATB\textsuperscript{0}/ASCT2 levels, it appeared that nearly two-thirds of the transporters had been effectively lost after 24 h of antisense RNA treatment (Fig. 3), coinciding closely with a 73% loss of the mRNA at the same time point (Fig. 2). Given the impact of apoptotic processes on plasma membrane integrity, driving forces (Na\textsuperscript{+} electrochemical gradients, etc.) for this transporter may have been compromised, making such
correlative relationships difficult to confirm. Regardless, the results suggest that the functional expression of the transporter was effectively diminished.

The original hypothesis on which these studies were based was that the molecular inhibition of \( \text{ATB}^{\text{ASCT2}} \) expression would lead to growth arrest primarily via glutamine limitation (6). Indeed, both transporter knockdown and glutamine starvation led to caspase-2 activation (Figs. 8 and 9). There is only one report that glutamine starvation activates caspase-2 in mammalian cells (29), but to the best of our knowledge, this is the first report of the relationship in human hepatic cells. Blunted glutamine delivery may contribute to apoptotic cell death when transporter expression is diminished, but it is clear from the data that glutamine deprivation is not alone sufficient for the observed effects of transporter suppression, and other compromised biological functions of \( \text{ATB}^{\text{ASCT2}} \) clearly account for the observed effects of transporter suppression, and other stress pathways are currently being pursued.

In conclusion, the studies presented here provide initial evidence that molecular inhibition of \( \text{ATB}^{\text{ASCT2}} \) expression leads to apoptotic cell death of an aggressively growing human liver cancer cell. Given that \( \text{ATB}^{\text{ASCT2}} \) is not expressed in normal hepatocytes, its further exploration as a selective target within the liver for treatment of HCC is justified.

**REFERENCES**